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Complete canine papillomavirus life cycle in pigmented lesions

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Abstract

Canine Papillomaviruses (CPVs) have been identified in various benign and malignant neoplastic skin disorders. The most frequent manifestations of CPV infections are classical warts and pigmented plaques. Although the etiology of canine oral papillomatosis is well established, knowledge about CPVs` role in the development of pigmented plaques remains vague. Indeed, as CPV DNA may frequently be found on clinically healthy canine skin, its mere detection in lesions cannot be regarded as a sufficient indicator of causality. Whether CPVs are actually active in pigmented plaques, a requirement for any conceivable involvement, is consequently an open question. To enquire such viral activity, two distinct clinical cases of canine pigmented lesions were evaluated in greater detail. The histological findings in the two cases supported the clinical diagnosis of pigmented viral plaques. Sequencing of amplified DNA from these lesions revealed the genomes of two novel CPV types, i.e. CPV9 and CPV14, both putatively belonging to the genus Chi. Furthermore, transcription and splicing of corresponding CPV mRNA could be shown by RT-PCR in the respective lesions. Finally, viral particles were detected by electron microscopy in homogenates as well as in nuclei of keratinocytes in pigmented lesions. In conclusion, the results link clinical signs of pigmented plaques to histological changes, the presence of CPV specific DNA, viral gene transcription, and the presence of viral particles in and from the lesions. Thus, the findings outline the entire replicative cycle of CPVs in pigmented plaques, which might help understanding the relationship between these viruses and the associated disorders.

Keywords: Papillomavirus; dog; pigmented plaques; RNA; virus particles

Introduction

Papillomaviruses are small double stranded DNA viruses with a circular genome of about 8 kilo base pairs packaged into an icosahedral capsid of around 50nm (Howley and Lowy, 2007). They are taxonomically grouped in the family Papillomaviridae that is expected to have a long evolutionary history due to its enormous genetic diversity. More than 200 types of papillomaviruses have already been identified in a broad variety of species, most of them in humans (Bernard et al., 2010). Papillomaviruses primarily infect keratinocytes and have been proven or proposed to play an important role in the development of benign and malignant tumors in mucous membranes and the keratinizing skin (Howley and Lowy, 2007).

Papillomaviruses depend very much on the lifecycle of keratinocytes which are their primarily host cells. The papillomavirus lifecycle can be divided into three phases. In the first phase an infection is established, which requires binding, entry, uncoating, nuclear transport and an initial amplification of the viral DNA in the nucleus. In the second phase, that might be very long, the viral gene expression is very low and the episomal papillomavirus genomes, which might be just some to a few hundred per cell, are copied in synchrony with the cellular DNA. In the third or late phase, which is initiated by yet unknown differentiation associated cellular factors, structural proteins are expressed and viral particles assemble in the nucleus of the terminally differentiating keratinocytes (Howley and Lowy, 2007, Egawa et al. 2012).

During the different phases of the papillomavirus lifecycle the viral gene expression differs significantly, consequently the viral Open Reading Frames (ORFs) were termed early (E) and late (L) ones. Thus the viral genome has an early and a late region, which usually have a polyadenylation site each. While the late genes L1 and L2, which code for the capsid proteins, are only expressed in the late phase of an infection, others namely E6 and E7 are rather constantly expressed at a low level. The proteins encoded by E1 and E2, which are the viral

players involved in replication, seem to be primarily needed in the first and third phase. Although the number of ORFs in a papillomavirus genome is usually only seven or eight, the number of expressed proteins exceeds this significantly due to intensive and alternative splicing. One of these spliced mRNAs codes for E1^E4 that is actually a late protein involved in packaging of the viral DNA (Howley and Lowy, 2007, Egawa et al. 2012).

As for their pathogenic role papillomaviruses are today well-studied pathogens of humans primarily in the context of cervix carcinomas and condylomas, which are a major health issue globally. Nevertheless there are also several benign and also malignant maladies of the keratinizing skin that are caused by or connected to papillomavirus infections, including the common warts, epidermoplasia veruciformis (EV) and carcinomas. Still, not only humans suffer from papillomavirus infections. Exophytic and endophytic warts, pigmented plaques and also in situ and invasive squamous cell carcinomas of dogs have been linked to papillomaviruses, and there is increasing evidence, that there is a causal relationship beyond oral papillomatosis. So far the genomes of nine canine papillomaviruses (CPVs) have been reported, as well as a couple of partial ones (Zaugg et al., 2005, Lange et al., 2010, Munday et al., 2011, Luff et al., 2012). All known CPVs can be allocated to three distinct papillomavirus genera, namely Lambda (CPVs 1 and 6), Tau (CPVs 2 and 7) and Chi (CPVs 3, 4, 5, 8 and 9) based on their L1 nucleotide sequences (Delius et al., 1994, Tobler et al., 2006, Yuan et al., 2007, Tobler et al., 2008, Lange et al., 2009, Bernard et al., 2010, Lange et al., 2012, Yuan et al., 2012). Based on the available data it appears, that all the pigmented plaques in dogs may be caused by Chi papillomaviruses.

Although there is good evidence that papillomaviruses do induce lesions in dogs, papillomavirus DNA is frequently found on clinically healthy skin of dogs and other species (Antonsson and Hansson 2002, Lange et al., 2011). If these findings amount to subclinical infections, then additional factors may be required to trigger apparent clinical papillomatosis.

Such might be genetic as in human EV, induced immunosuppression as in organ recipients, acquired like in HIV patients or due to exposure to other external stimuli like UV light or chemicals (zur Hausen 2009, Heard 2011). Several reports from the past years support this hypothesis, however, the sole identification of viral DNA in lesions leaves room for debate whether or not the virus is active and thus putatively playing an active role or not (Le Net et al., 1997, Callan et al., 2005, Favrot et al., 2005, Goldschmidt et al., 2006). To determine if there is ongoing virus activity in plaque-like pigmented lesions, two cases of papillomatosis involving novel Chi papillomaviruses were analyzed in detail, including clinical assessment, histology, PCR, molecular cloning, sequence analysis, phylogenetic analysis, transcription analysis and electron microscopy.

Materials and Methods

Samples

Two dogs were included in this study, which were both presented at the veterinary teaching hospital of the University of Zurich. The first dog was a ten year old female castrated Golden retriever presenting with several hyperpigmented, plaque-like, non-pruritic lesions on the right dorsal foreleg. Most of the lesions were pedunculated, giving the lesion a scaly appearance (Fig. 1). Six years before the dog had been diagnosed with low-grade lymphoma. After a short period of a higher dose in the beginning, the dog received 0,3mg/kg prednisolone every other day and 0,05mg/kg chlorambucil daily as therapy.

The second dog was an eight-month-old intact black pug showing several pigmented plaques on the abdomen, right medial thigh, right knee and neck. The lesions were non-pruritic. The dog had previously been presented because of a subclinical extra hepatic porto-systemic shunt and a generalized juvenile demodicosis, both being diagnosed 4 month earlier. The

demodicosis was cured at the time, the pigmented plaques developed; the porto-systemmic shunt was closed by cellophane ligation 3 month earlier.

Initially in each of the cases one 6mm punch biopsy for histopathological examination and one cytobrush sample for microbiological assessment were taken from the lesions. The cytobrush was moistened with sterile 0.9% NaCl solution, rubbed for thirty seconds on the affected area and stored in an 1.5ml Eppendorf tube at -20°C until DNA extraction. The tissue samples were fixed in 4% buffered formalin. After embedding in paraffin, 4 µm sections were stained with haematoxylin and eosin (HE) for histopatholgical examination.

Upon a second visit samples for transmission electron microscopy and negative stain as well as for RNA assessment were taken. To preserve the RNA the respective samples were placed in RNA later (Sigma-Aldrich) and stored according to the protocol.

Electron microscopy

The biopsies were fixed with 2.5% glutaraldehyde in 0.1M Na/K-phosphate, pH 7.4, at 4°C for 1 h, kept in 0.1M Na/K-phosphate at 4°C overnight, post-fixed with 1% osmium tetroxide in 0.1M Na/K-phosphate at 4°C for 1 h, dehydrated in a graded ethanol series starting at 70% and, after two changes in acetone, embedded in epon. 50 – 60 nm thick sections were stained with uranylacetate and lead citrate, and analyzed in a transmission electron microscope (CM 12, Philips, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

For negative staining, the biopsies were cut with a razor blade into small cubes, and after three cycles of freezing and thawing, centrifuged in an eppendorf centrifuge at maximal speed. From the supernatant, virus particles were adsorbed for 10 min to carbon coated parlodion films mounted on 300 mesh/inch copper grids (EMS; Fort Washington, PA, USA),

washed once with distilled water, and stained with 2% phosphotungstic acid (Sigma Aldrich, Gilingham- Dorset, UK), pH 7.0, at room temperature for 1 min. Specimens were analyzed in a transmission electron microscope CM 12, equipped with a CCD camera Ultrascan 1000 at an acceleration voltage of 100 kV.

Identification, Amplification and cloning of genomes

Total DNA of 25 mg tissue samples was isolated using a DNeasy extraction kit (Qiagen) according to the manufacturer's recommendations. For initial identification of host and PV DNA in the samples GAPDH f/r, CP4/CP5 and canPV/FAP64 PCR assays were applied (Iftner et al., 2003, Lange et al., 2011). PCR products were determined (Microsynth) by cycle sequencing using an ABI 377 sequencer (Applied Biosystems) and compared to the NCBI database (BLAST X). For cloning purposes one microliter of the DNA extract was used for rolling circle amplification (RCA) (Rector et al., 2004), using a TempliPhi Amplification kit (General Electrics Biosciences). Slight modifications were applied to the protocol supplied by the manufacturer (Lange et al., 2009). Amplified DNA from case one (CPV14) was cloned into the *Bam*HI, *Eco*RI or *Xba*I site of pBluescript II KS+ (Stratagene), while the DNA amplified from case two was cloned into the *Sal*I site (CPV9) using standard procedures. The cloned DNA sequences of the PV candidates were determined (Microsynth) and primary sequences were assembled using Contigexpress software (Vector NTI Informax, Invitrogen). The nucleotide sequence data of CPV14 were deposited in GenBank under accession no. JQ701802.

Sequence analysis

The coding sequences for the E6, E7, E1, E2, L2 and L1 proteins from the CPVs, feline

156 papillomaviruses (FdPVs) and bovine papillomavirus 1 (BPV1) were translated and aligned
157 by using MAFFT (Katoh and Toh, 2008) before being back-translated to DNA sequences.
158 The six sets of aligned nucleotide sequences representing the six sets of protein sequences
159 were shortened by using GBLOCK (version 0.91b; http://molevol.cmima.csic.es/castresana/)
160 (Castresana et al., 2000) and combined to a single multiple sequence alignment (MSA) by
161 concatenating the sequences from each virus. The optimal model of DNA evolution was
162 evaluated for best fit of the data set using MODELTEST (version 1.4.4;
163 http://darwin.uvigo.es/; default settings) (Posada 1998). Bayesian phylogeny was inferred
164 using MRBAYES (version 3.2; http://mrbayes.csit.fsu.edu/; Markov Chain Monte Carlo with
165 GTR substitution matrix, variable gamma rates, invariant sites, two runs four chains of
166 10,000,000 generations), and displayed with FIGTREE (1.3.0 http://tree.bio.ed.ac.uk/)
167 (Drummond et al., 2007). Pairwise sequence alignments were performed using NEEDLE
168 (EMBOSS, nucleotide matrix: DNAFULL, amino acid matrix: BLOSSUM62). Splice sites
169 were predicted by the on-line version of NETGENE2
170 (http://www.cbs.dtu.dk/services/NetGene2/) (Brunak et al., 1991).
171

172 **Detection of virus specific mRNA**

173 Total RNA of 25 mg tissue samples was isolated using a NucleoSpin RNA II RNA extraction
174 kit (Machery Nagel) according to the manufacturer's recommendations. To get rid of putative
175 remnant DNA the RNA was afterwards again digested with DNase A (Roche), 1µl DNase
176 was added to 50 µl sample and incubated for 15 min at 37°C and afterwards 10 minutes at
177 75°C. A Reverse Transcription System reverse transcription kit (Promega) was used
178 according to the protocol to produce cDNA with random primers. To control the success of
179 the DNA digestion, one sample with and one sample without reverse transcriptase (RT) each
180 was processed.

To test for RNA transcription, primer combinations amplifying fragments of the main transcripts, E6, E7, E1, E2, E1^E4, L2 and L1 were constructed (supplement 2). Except for E4 all primers were designed to amplify regions were no splicing was expected. The primers for E1^E4 PCR however were designed to identify donor and acceptor splice sites of the E1^E4 mRNA. PCR was performed on cDNA with and without RT, sterile water and DNA extract of lesional tissue.

To determine the splice sites the E4 PCR product from the cDNA was extracted from the gel using a QIAEX II kit (QIAGEN) and sequenced (Microsynth). The sequence of the putative E4 of CPV14 was included in the file JQ701802. The putative E4 sequence information of CPV9 was deposited separate in GenBank under accession no. JQ701801.

Results

Clinical development

The course of the papillomavirus infections after presentation varied significantly. The lesions in the Golden retriever resolved entirely within few weeks despite of continued corticosteroid treatment. In the pug on the contrary more lesions developed over the following months, but no approach of treatment was attempted.

Histology

In case of the included pug the histological examination revealed papillated hyperplasia and hyperpigmentation of the epidermis with large clumped keratohyalin granules. A rete ridges formation was abundant and single scattered clear cells reminiscent of koilocytes in the stratum granulosum (Fig. 1C). A diagnosis of canine viral pigmented plaques was made.

The histological sections from the Golden retriever showed papillated hyperplasia and hyperpigmentation of the epidermis with large clumped keratohyalin granules. They were also found to contain disseminated apoptotic cells and some scattered clear cells reminiscent of koilocytes in the stratum granulosum (Fig. 1D). A papilloma was diagnosed.

DNA

In the attempt to determine whether the samples contain papillomavirus genomes, DNA was successfully amplified from the samples of the two cases by PCR. A comparison of the sequenced PCR products with the NCBI database revealed the sequence of a novel canine papillomavirus (CPV14) in the samples taken from the Golden retriever. The sequence obtained from the samples taken from the pug was found to be identical with the sequence of a very recently discovered papillomavirus (CPV9). The sequencing of cloned RCA product of CPV14 revealed a papillomavirus genome of 7784 nucleotides. Its entity was verified by direct sequencing of the RCA product.

In case of both CPVs the characteristic PV open reading frames (ORFs) E1, E2, E4, E6, E7, L1 and L2 and two non-coding regions (NCR) were identified in the genome. Comparison with the other PVs on the nucleotide level using pairwise alignments (Needle, EMBOSS, http://emboss.ch.embnet.org/wEMBOSS/) identified CPV3 as the closest relative of CPV9 (72% identities) and CPV8 as the closest known to CPV14 (74% identities) based on the L1 ORF (Table 1). CPV9 has a GC content of 51%, CPV14 of 53%.

Characteristic papillomavirus motifs were identified on the genomic sequences. Nine putative E2 binding sites (ACC-N_6-GGT) could be located on the CPV9 sequence, six in the NCR, one within the E2/E4 ORF, one in the L1 and one in the L2 ORF. Flanked by E2 binding sites dyad symmetry repeats (TTGTTGTTAACAACAA) in a modified form were found 101nt

upstream of the E6 ORF. Poly A signals (AATAAA) were identified at the ends of the E1 and L1 ORFs as well as at the start of the L2 ORF, also one putative SP1 binding site (GGCGGG) in the L1 ORF and 11 putative NF1 binding sites (GCCAA). The E6 amino acid sequence contains two metal binding motifs ($CX_2CX_{28-30}CX_2C$) starting at amino acids 25 and 98, the E7 one starting at amino acid 54. The amino acid sequence of E7 also contains a putative pRB-binding motif (LXCXE). A modified ATP helicase-binding motif (GPPDTSKS) is present on the predicted E1 amino acid sequence. The CPV14 genomic sequence contains eleven putative E2 binding sites, five of them in NCR, one within the E2/E4 ORF, one in the L1 and two in the L2 ORF. Located in the NCR, 98nt upstream of the E6 ORF flanked by E2 binding sites dyad symmetry repeats were identified. Poly A signals were identified within the E1 and at the ends L1 ORF. One putative AP1 binding site in the E2/E4 ORF and 12 putative NF1 binding sites were also found. Metal binding motifs were identified within the E6 amino acid sequence starting at amino acids 25 and 97 as well as in the E7 amino acid sequence starting at amino acid 54. A putative pRB-binding motif is also present in the encoded E7 amino acid sequence. An ATP helicase-binding motif is present on the predicted E1 amino acid sequence.

Phylogeny

To allocate the two viral genomes in a phylogenetic context, they were compared to the other canine and feline papillomaviruses and a bovine papillomavirus 1 sequence. In a phylogenetic tree based on the E6, E7, E1, E2, L1 and L2 regions both CPVs appear in the branch of the Chi papillomaviruses (Fig. 2). While CPV9 clusters together with CPVs 3, 4, 5 and 11, CPV14 clusters together with CPVs 8 and 10. CPVs 9 and 14 share more than 60% of identities with the described Chi papillomaviruses CPV3, 4, 5 and 8 as well as with CPVs 10 and 11. When compared on the amino acid level CPV9 shows between 78.1 and 86.8%

similarity with CPVs 3, 4, 5 and 11 in the E6 sequence and with the same viruses between 89.5 and 96% similarity in the E7 sequence. CPV14 shows between 74.8 and 80.6% similarity with CPVs 10 and 8 in the E6 sequence and 88.5 and 91.4% similarity in the E7 sequence (supplement 1).

RNA & Splicing

To determine whether the viral genes were transcribed in the lesional tissue, total RNA was extracted from fresh samples, reverse transcribed into cDNA and analyzed by PCR. Different PCRs with specific primer sets were applied to amplify cDNA of the host GAPDH mRNA, as well as of the viral mRNAs of L2, L1, E6, E7, E1, E2 and E1^E4 of CPV9 or CPV14 (supplement 2). The RT-PCR revealed amplimers of expected sizes. However, no product was produced if the reverse transcriptase was omitted in the cDNA reaction (Fig. 3).

Using the E1^E4 transcript specific primers, no PCR product of the expected size (3030 and 3159 bp, respectively; see supplement 2) was produced from the DNA extract of the lesions. A side product of unknown origin was amplified with the CPV9 E1^E4 specific primer of about 150 bp was visible in the DNA- and the RT+-sample. The sequencing of the E1^E4 RT-PCR product from the RNA samples revealed a joined sequence of the first 22 nucleotides of the E1 ORF at genome position 992 and the predicted E4 ORF from position 3382 in case of CPV9 and the first 22 nucleotides of the E1 ORF at genome position 851 and the predicted E4 ORF from position 3259 in case of CPV14. These sites are in agreement with the splice donors (0.66 and 0.92 confidence for CPV9 and CPV14, respectively) and the splice acceptors (0.14 and 0.19 confidence for CPV9 and CPV14, respectively) predicted by NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/).

Electron Microscopy

Examination of the affected epidermis by transmission electron microscopy revealed numerous, uniform round particles in nuclei of keratinocytes in the basal and granular layer of both dogs (Fig. 4 A-B). Extracts obtained after freezing and thawing contained particles of ~50 nm in diameter showing the characteristic morphology of papillomavirus after negative staining with Na-phosphotungstic acid (Fig. 4 C-D).

Discussion

Pigmented plaques and putatively associated skin alterations are infrequently seen in dogs of various ages and have been proposed to be papillomavirus induced (Nagata et al., 1995, Narama et al., 2005, Tobler et al., 2006, Tobler et al., 2008, Lange et al., 2009, Munday et al., 2011, Lange et al., Luff et al., 2012, 2012, Yuan et al., 2012). The question whether viruses are present and may be causing the observed lesions has been addressed in various ways that can be subsumed under two categories of approaches. On the one hand characteristic changes indicative of papillomavirus infections have been described and even viral particles have been found in pigmented plaques using a pathological approach (Nagata et al., 1995, Narama et al., 2005). On the other hand DNA sequences of various Chi-papillomavirus types or putative types have been retrieved from pigmented plaques using rather a molecular biological approach (Lange et al., 2009, Yuan et al., 2012). Both approaches however have their strengths and weaknesses. While the visualization of macroscopic and microscopic changes and even viral particles clearly proves a pathology and virus production it fails to determine the pathogen. The identification and determination of viral DNA allows a detailed analysis of the genetics of a putative pathogen, makes some predictions about its biology possible and is very helpful to address the prevalence, but still does not tell much about the activity or role of

it in the lesion. Especially a prevalence of virus DNA in nonlesional skin in many species has to be kept in mind.

The two cases analyzed here resemble typical lesions associated with Chi papillomaviruses, clinically as well as from the histopathological point of view. The flat pigmented warts as seen in case two appear to be identical with the lesions associated with CPVs 4 and 8 (Tobler et al., 2008, Lange et al., 2012). Similarly the pigmented scale like lesions of case one are indistinguishable from those found in the cases associated with CPVs 3 and 5 although no pigmented plaques were apparent in case one while they were in dogs infected with CPVs 3 or 5 (Tobler et al., 2006, Lange et al., 2009). The clinical diagnosis of papillomatosis was confirmed on the one hand by some characteristic histopathological alterations and the PCR based discovery of papillomavirus DNA in the lesional skin. The history of both cases also involved additional factors that are known amplificators of papillomavirus infections as they interfere with the immune system. In case of the Golden retriever this is the long time application of the immunosuppressant cortisone. In case of the pug it is the extra hepatic porto-systemic shunt, although the mechanisms by which such a defect influences the immune system are only partly understood (Koblik and Hornof, 1995). If, and if so how these putative amplificators contributed to the clinical lesions remains open. The development of the two cases suggests, that the low dose cortisone may have only partly been promoting the onset of the clinical papillomatosis as it spontaneously resolved. The shunt-induced immunosuppression thus seems to have had a longer lasting effect. However other factors including the virus itself may have been responsible for the development of the lesions.

To determine if the virus is actually going to a full lifecycle in the lesions or whether it's DNA is rather there in a silent or latent state viral gene expression was evaluated. In this context the expression of the seven viral transcripts E1, E2, E4, E6, E7, L1 and L2 indicates, that infected cells of different stages are present, as the expression of certain mRNAs is

326 regulated according to the stage in the lifecycle of the virus and its host cell. The terminal
327 stage of a papillomavirus lifecycle is the assembly and packaging of viral particles in its
328 nucleus before the cell dies (Howley and Lowy, 2007). The assembled particles in the nuclei
329 could be shown by transmission electron microscopy and viral particles could also be visualized
330 using negative stain techniques (Fig. 4). This strongly indicates, that the entire virus life cycle
331 of CPV9 and CPV14, respectively, must have been completed in the respective lesions.

332 Splicing is known to be an important posttranscriptional process for papillomavirus gene
333 expression and is likely to occur in all papillomaviruses. Although putative donor and
334 acceptor sites can be predicted to a certain extent, the actual splicing products have to be
335 confirmed experimentally. The presented experiments determined splice donor and acceptor
336 sites of the E4 mRNAs of CPV9 and CPV14.

337 The Phylogenetic analysis of the CPV sequences indicates at least two mayor branches
338 containing three or more species within the genus Chi genus of papillomaviruses (Fig. 2). One
339 branch contains the CPVs 3, 5, 9 and 11 that cluster well together and which would fulfill the
340 criteria of one taxonomic species (>70% identities). CPV4 Appears to be in that branch as
341 well, but it would represent an own species. However, in case of the second branch, which
342 includes CPVs 8, 10 and 14 applying the species definition is not that easy. When looking at
343 CPVs 8 and 10 independently, they would represent two different species (<70% identities),
344 whereas CPV14 seems to be in between fulfilling the criteria for both (table 1). Such
345 difficulties with the current definitions arise occasionally as more and more papillomavirus
346 sequences become available and may make some revisions necessary in the future.

347 Interestingly the degree of similarities in the encoded amino acid sequences of E6 and E7
348 resemble very well the phylogenetic tree. As proteins encoded by the E6 and the E7 gene are
349 supposedly the products that have the most influence on papillomavirus pathologies this
350 indicates some biological relevance (supplement 1). A huge amount of clinical data would

however be needed to determine whether the clinical outcome of infections with different Chi papillomaviruses may differ due to the virus type involved or due to host or other factors.

Proving causality between papillomavirus infections and associated lesions has always been a challenge. Yet, demonstration of not only presence but also active gene expression and completion of the viral life cycle of CPV9 and CPV14, respectively, in those lesions brings us at least one step closer to the goal. Although that does not prove causality, these findings do support such a hypothesis.

In summary two novel papillomaviruses have been found, sequenced, analyzed in a phylogenetic context and shown to exist and express their genes in lesions frequently described as papillomavirus-associated.

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Figure captions:

Figure 1: Pigmented plaques on the abdomen of a pug from a macroscopic (A) and microscopic perspective (HE, 10x objective). Histology reveals hyperplasia, hyperpigmentation and rete ridges formation (C). Golden retriever presenting with pigmented plaque and scale like lesions on a foreleg (B). Under the microscope (HE, 10x objective) hypergranulosis, hyperkeratosis and apoptotic cells are visible (D).

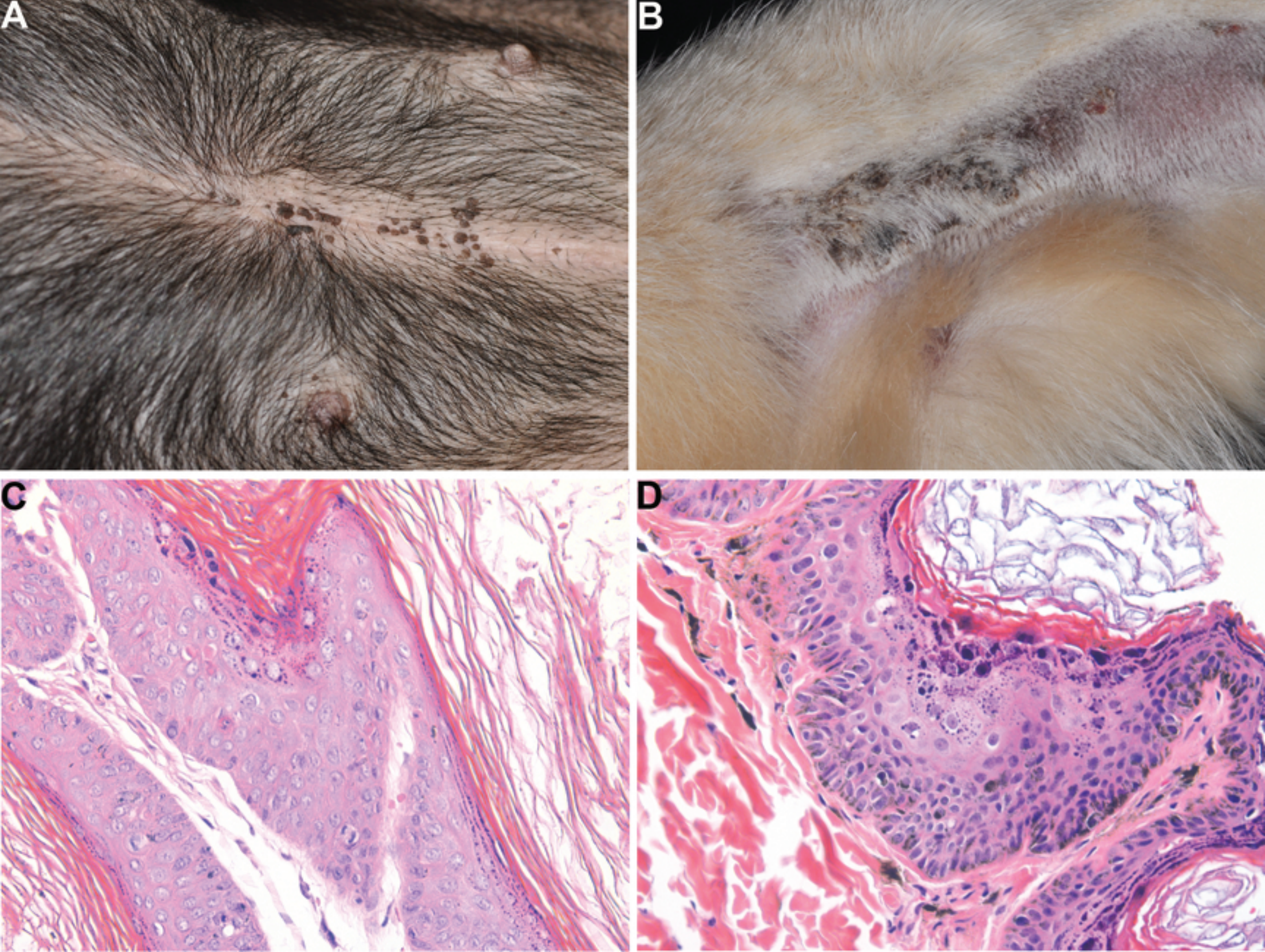
Figure 2: Bayesian phylogenetic tree based on the alignment of E6, E7, E1, E2, L1 and L2. The PV-types (with GenBank accession numbers) included are: bovine - BPV1 (X02346); canine - CPV1 (L22695), CPV2 (AY722648), CPV3 (NC_008297), CPV4 (NC_010226), CPV5 (FJ492743), CPV6 (FJ592744), CPV7 (FJ492743), CPV8 (HM796884), CPV9 (NC_016074), CPV10 (NC_016075), CPV11 (NC_016076) and CPV14; feline - FdPV1 (NC_004765) and FdPV2 (EU796884). Numbers at internal nodes represent the posterior probability support values.

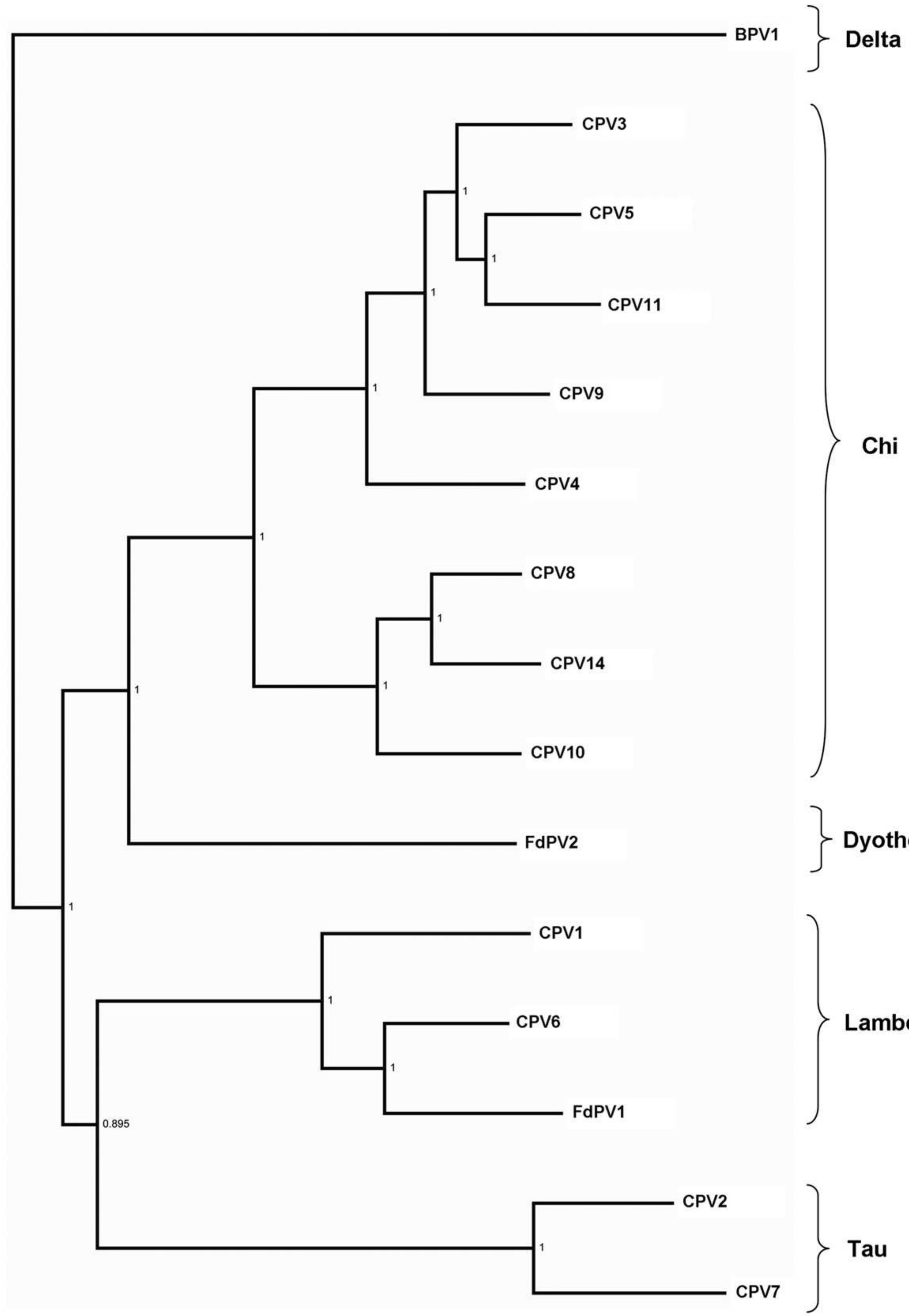
Figure 3: Agarose gel electrophoresis of the PCR results obtained from the Golden retriever (A) and the pug (B). PCR assays for seven characteristic papillomavirus genes L2, L1, E6, E7, E1, E2 and E4 of CPV14 (A) and CPV9 (B) and for the host gene GAPDH. Analyzed samples were total RNA extract with reverse transcription (RT+), total RNA extract without reverse transcription (RT-), total DNA extract (DNA) and elution buffer AE (Buffer). One hundred base pair ladder with fragment sizes from 100bp to 1500bp (in 100bp steps) and a 2072bp band, indicates product sizes (Marker).

Figure 4: Transmission electron micrographs of the lesional epidermis of the pug (A) and the Golden retriever (B) showing the nuclei of keratinocytes full with uniform round electrodense particles; bar 0.5µm. Extracts of lesional tissue from the pug (C) and the Golden retriever (D) showing round electrodense particles in negative stain electromicroscopy; bar 50nm.

Table 1
Comparison of the L1 ORFs at the nucleotide level, values in percentage identities

	CPV1	CPV2	CPV3	CPV4	CPV5	CPV6	CPV7	CPV8	CPV9	CPV10	CPV11	CPV14
CPV1		57	54	56	57	69	58	60	58	56	55	58
CPV2	57		55	53	56	56	70	57	55	57	54	55
CPV3	54	55		64	73	53	54	62	72	62	73	61
CPV4	56	53	64		66	56	55	63	67	62	64	63
CPV5	57	56	73	66		56	55	62	71	63	73	61
CPV6	69	56	53	56	56		56	57	54	56	55	55
CPV7	58	70	54	55	55	56		57	55	55	55	56
CPV8	60	57	62	63	62	57	57		64	71	62	74
CPV9	58	55	72	67	71	54	55	64		64	71	61
CPV10	56	57	62	62	63	56	55	71	64		63	68
CPV11	55	54	73	64	73	55	55	62	71	63		61
CPV14	58	55	61	63	61	55	56	74	61	68	61	


A
B
C
D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Delta
CPV3
CPV5
CPV11
CPV9
CPV4
CPV8
CPV14
CPV10
Chi
FdPV2
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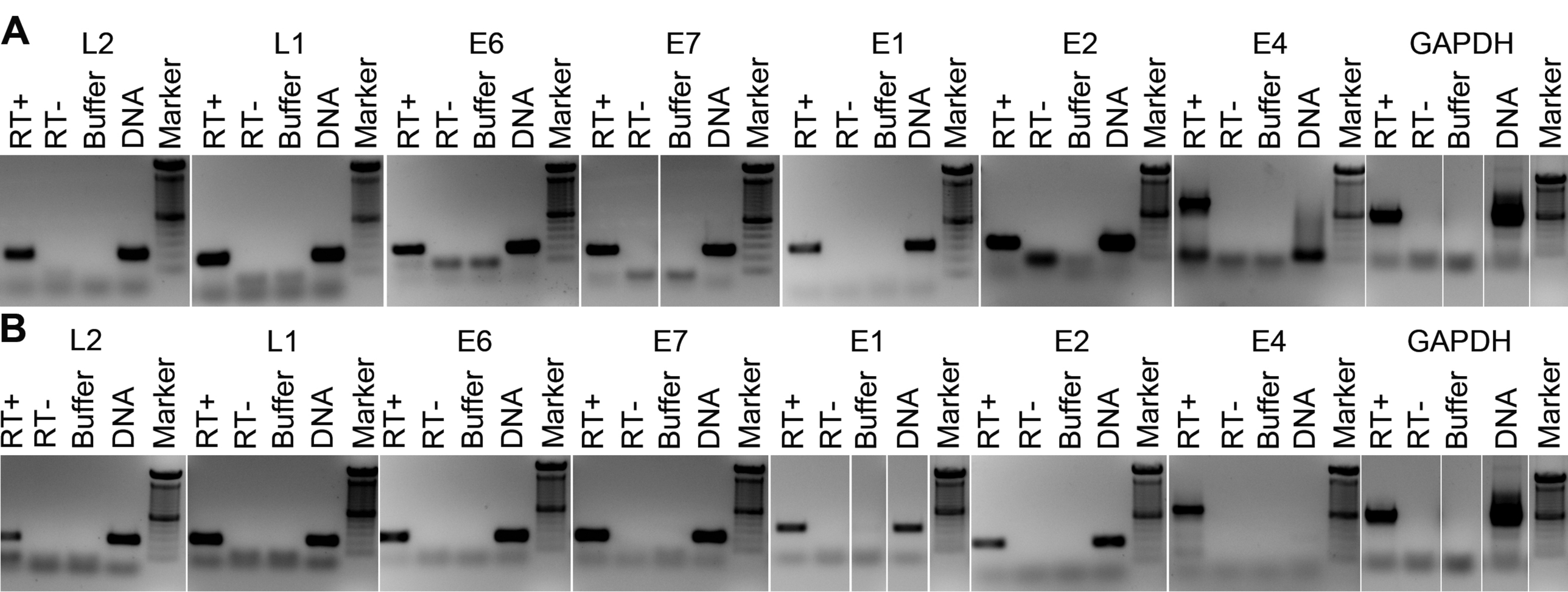
Lambda
CPV2
CPV7
Tau
1
0.895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L1
E6
E7
E1
E2
E4
GAPDH
RT+
RT-
Buffer
DNA
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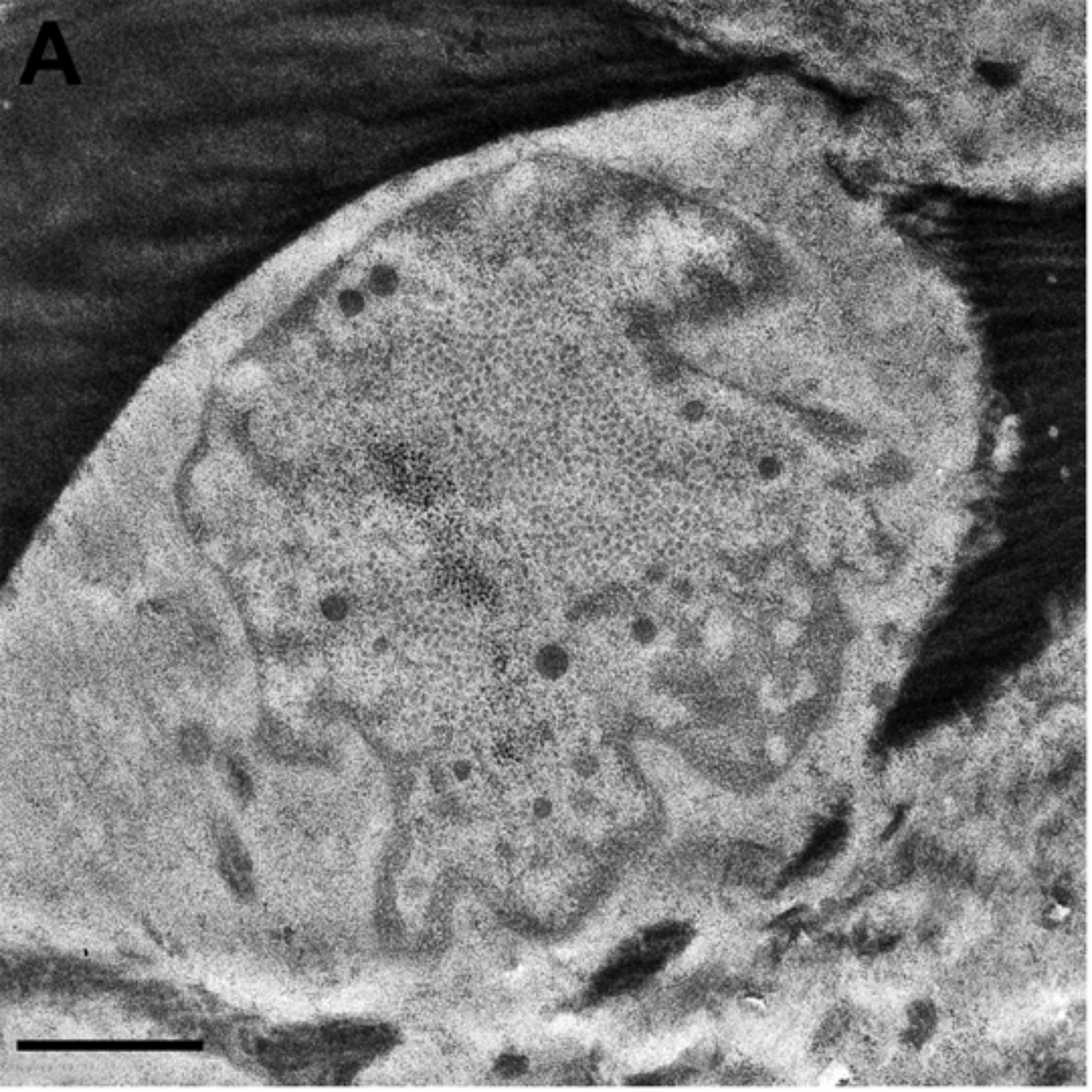
L1
E6
E7
E1
E2
E4
GAPDH
RT+
RT-
Buffer
DNA
Marker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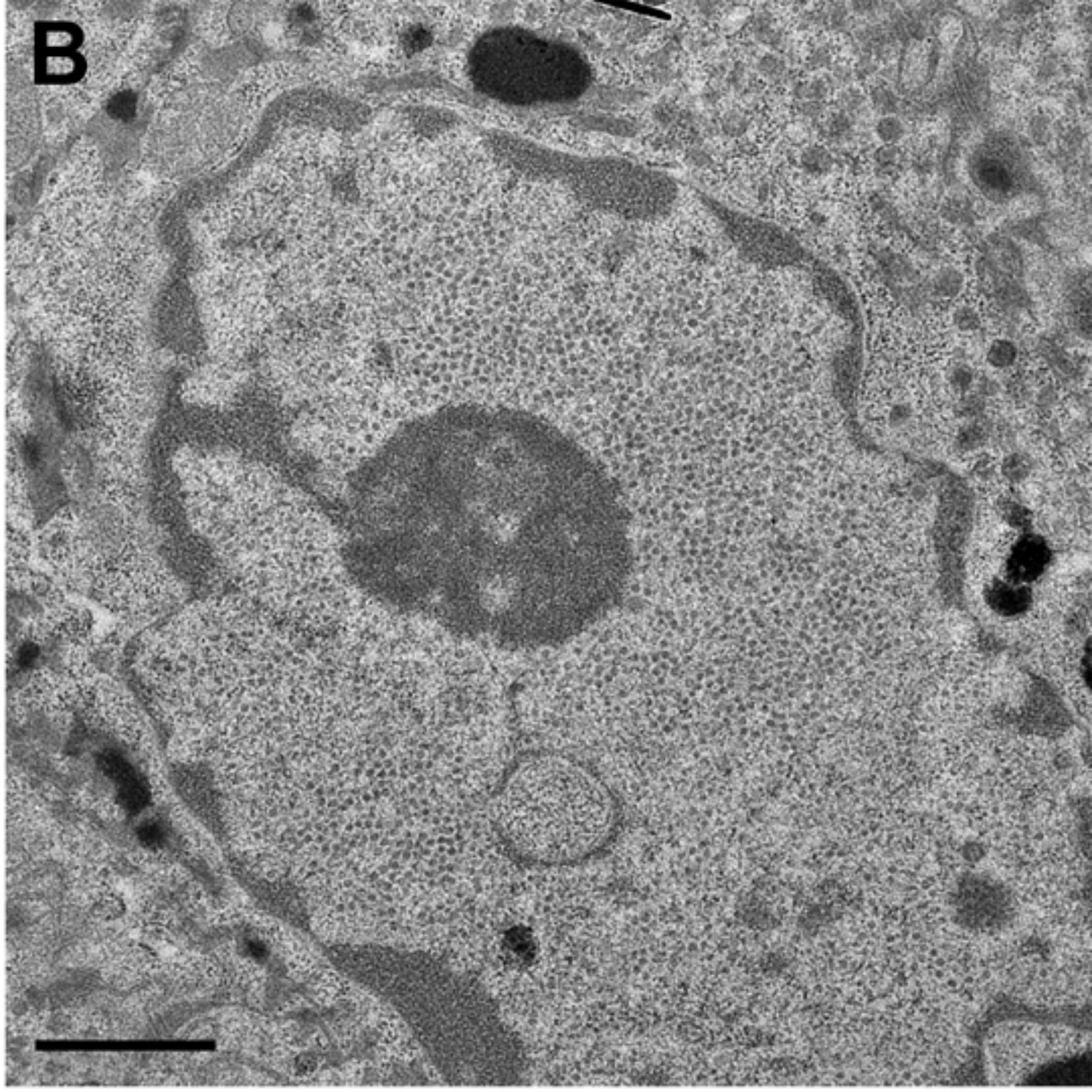
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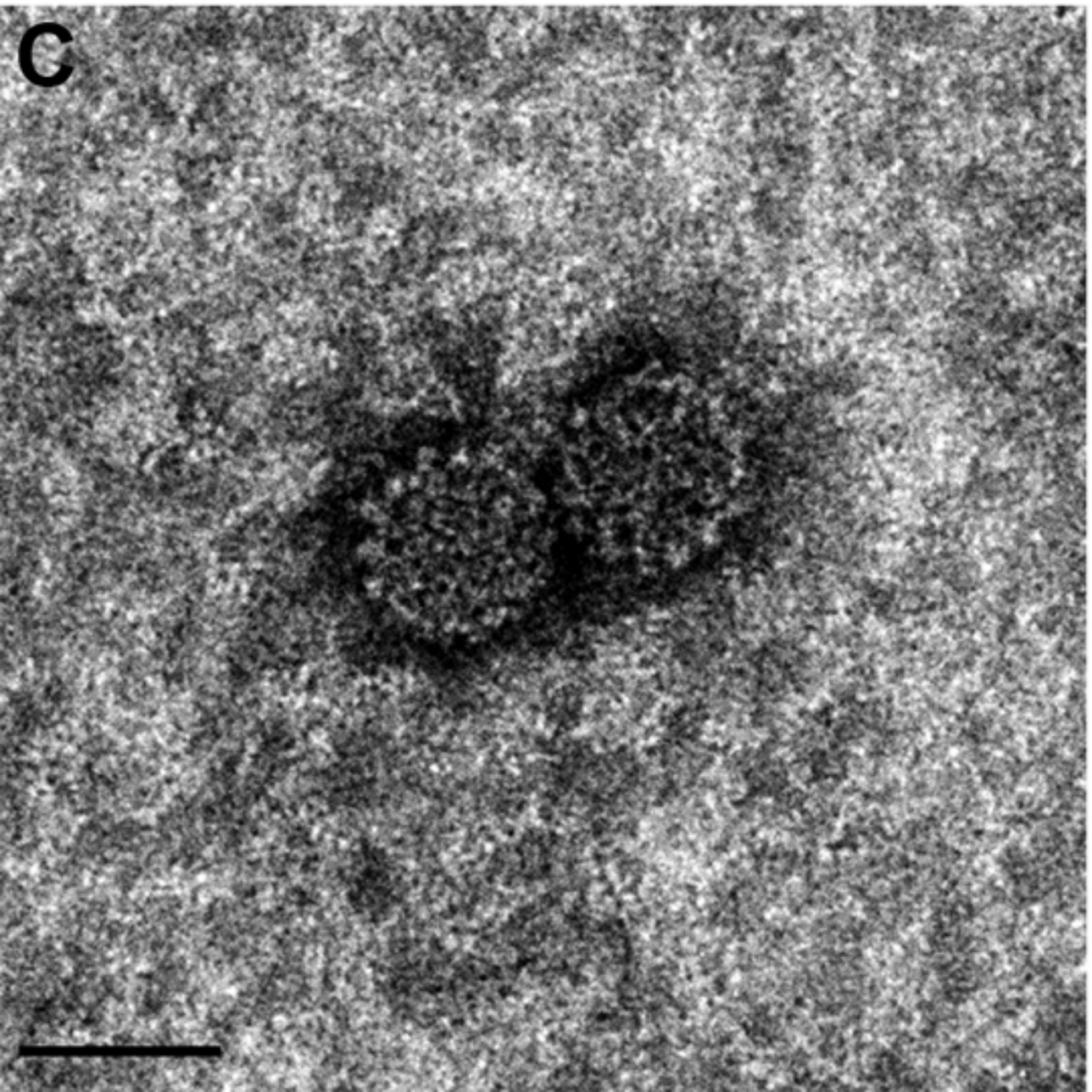
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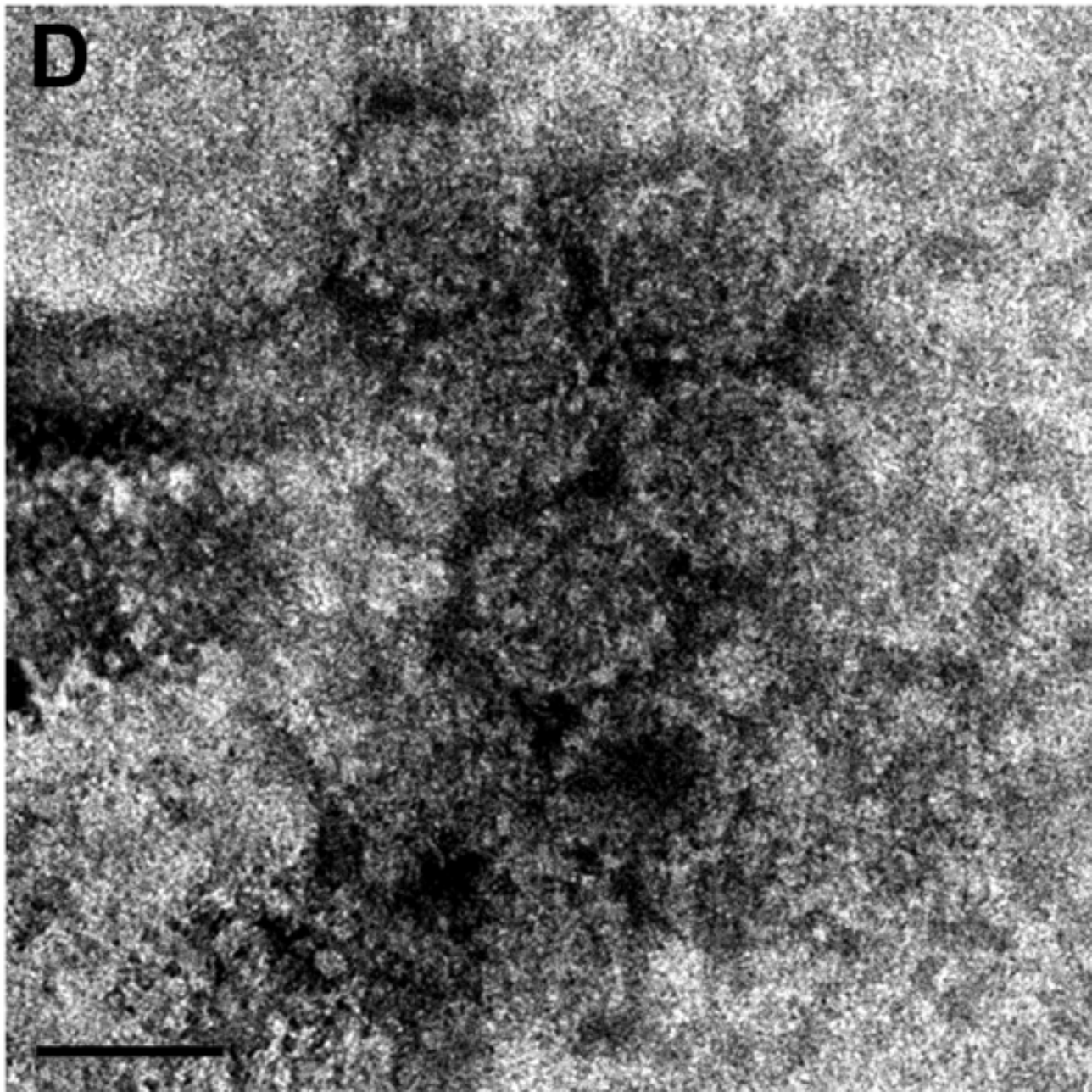
D

Identity [E1.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	38.4	34	38.4	39.3	39.4
cpv01	38.4	100	44.1	41.4	42	42.3
cpv02	34	44.1	100	42.6	41.6	42.1
cpv03	38.4	41.4	42.6	100	71.3	78.1
cpv04	39.3	42	41.6	71.3	100	75.4
cpv05	39.4	42.3	42.1	78.1	75.4	100
cpv06	41.6	63.4	44.9	43	44.5	44
cpv07	33	43	70.1	40.2	39.3	40.3
cpv08	37.7	43.4	40.6	56	58.6	59.6
cpv09	39	41.1	42.6	74.3	73	83
cpv10	37.5	43.9	41.7	57.5	58.2	59.8
cpv11	39.4	41.2	41.7	79	75.4	87.3
cpv14	39.2	44.8	40.4	56.5	58.1	57.3
fdpv1	40.5	60.9	42.9	43	46	43.9
fdpv2	38.4	48.7	43.6	44.2	47.6	46.1

Similarity [E1.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	61.6	59.4	61.7	61	63
cpv01	61.6	100	68.8	67.2	66	68.6
cpv02	59.4	68.8	100	66.1	65.9	66.3
cpv03	61.7	67.2	66.1	100	86.3	90.3
cpv04	61	66	65.9	86.3	100	89.5
cpv05	63	68.6	66.3	90.3	89.5	100
cpv06	64	81.4	70.4	64.5	67.2	67.7
cpv07	61	66.8	85.2	65	63.2	65.8
cpv08	62.7	70.7	65.3	77.6	80	81
cpv09	61.2	66.4	64.7	87	87.6	92.9
cpv10	62.8	71.7	66.3	77.7	79.1	80.1
cpv11	62.7	68.7	66.4	90.5	90.5	96.5
cpv14	61.7	69.1	65.9	76.5	79.4	79.5
fdpv1	63.6	80.7	67.5	66.5	68.8	68.5
fdpv2	61	71	69.5	66.9	69.9	69.8

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	41.6	33	37.7	39	37.5	39.4	39.2
	63.4	43	43.4	41.1	43.9	41.2	44.8
	44.9	70.1	40.6	42.6	41.7	41.7	40.4
	43	40.2	56	74.3	57.5	79	56.5
	44.5	39.3	58.6	73	58.2	75.4	58.1
	44	40.3	59.6	83	59.8	87.3	57.3
	100	41.7	45.2	46	45.8	44.8	45.9
	41.7	100	40.9	39.8	41.4	39.7	41
	45.2	40.9	100	58	72.4	58.2	79.4
	46	39.8	58	100	59.1	79.8	57.5
	45.8	41.4	72.4	59.1	100	59.3	71.3
	44.8	39.7	58.2	79.8	59.3	100	59.1
	45.9	41	79.4	57.5	71.3	59.1	100
	71.3	39.6	45	42.8	44.3	43.7	47.2
	51.7	43.2	48.2	45.2	46.2	45	49.6

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	64	61	62.7	61.2	62.8	62.7	61.7
	81.4	66.8	70.7	66.4	71.7	68.7	69.1
	70.4	85.2	65.3	64.7	66.3	66.4	65.9
	64.5	65	77.6	87	77.7	90.5	76.5
	67.2	63.2	80	87.6	79.1	90.5	79.4
	67.7	65.8	81	92.9	80.1	96.5	79.5
	100	67.1	68.6	67.2	69	67.9	67.3
	67.1	100	67.4	64.7	67.8	64.6	66.6
	68.6	67.4	100	78.5	89.8	80.8	93.1
	67.2	64.7	78.5	100	79.5	91.4	77.7
	69	67.8	89.8	79.5	100	80.5	89
	67.9	64.6	80.8	91.4	80.5	100	79
	67.3	66.6	93.1	77.7	89	79	100
	86.9	65.4	67.8	66.8	68.3	66.9	67.7
	74	68.6	71.7	68.6	71.2	70.1	71.9

fdpv1	fdpv2
40.5	38.4
60.9	48.7
42.9	43.6
43	44.2
46	47.6
43.9	46.1
71.3	51.7
39.6	43.2
45	48.2
42.8	45.2
44.3	46.2
43.7	45
47.2	49.6
100	50.8
50.8	100

fdpv1	fdpv2
63.6	61
80.7	71
67.5	69.5
66.5	66.9
68.8	69.9
68.5	69.8
86.9	74
65.4	68.6
67.8	71.7
66.8	68.6
68.3	71.2
66.9	70.1
67.7	71.9
100	69.7
69.7	100

Identity [E2.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	30.4	25.5	28.7	29.5	27.1
cpv01	30.4	100	27.1	29.9	31.8	32.7
cpv02	25.5	27.1	100	29.8	29.1	28.6
cpv03	28.7	29.9	29.8	100	49.9	59.3
cpv04	29.5	31.8	29.1	49.9	100	47.7
cpv05	27.1	32.7	28.6	59.3	47.7	100
cpv06	30.6	51.6	29.3	32.4	33	31.6
cpv07	27.3	28.6	50.8	28.9	27.4	30.2
cpv08	25.8	30.7	28.4	41.5	40.2	41
cpv09	28.6	30.8	28	55.6	46.9	57.7
cpv10	27.2	32.5	26.3	39.7	40.8	39.4
cpv11	29	30.1	29	56.2	49.2	66.3
cpv14	25.5	30.1	27.8	38.2	36.7	41
fdpv1	31.4	51.3	29.9	31.6	32.7	32.6
fdpv2	28.4	31.1	34.7	31.5	31.4	33.4

Similarity [E2.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	53.7	45.1	47.5	47.9	46.7
cpv01	53.7	100	43.7	49.9	52.1	53.2
cpv02	45.1	43.7	100	53.4	52	52.6
cpv03	47.5	49.9	53.4	100	69.9	76.2
cpv04	47.9	52.1	52	69.9	100	68
cpv05	46.7	53.2	52.6	76.2	68	100
cpv06	51.7	73.2	47.2	52	53.4	52
cpv07	47.2	47.3	70.4	47.5	48.7	51
cpv08	43.2	47.6	52.7	64.5	60.3	62.2
cpv09	49.6	54.1	49.4	76.2	69.4	78.1
cpv10	44.9	48.5	48.2	60.3	58	62.4
cpv11	50.3	53	53.9	77.7	68	82.9
cpv14	43.8	47.8	49.2	59.9	58.4	62
fdpv1	51.9	73.2	45.6	49.5	52.4	52.5
fdpv2	46.9	47.5	55	48.1	47.6	52.1

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	30.6	27.3	25.8	28.6	27.2	29	25.5
	51.6	28.6	30.7	30.8	32.5	30.1	30.1
	29.3	50.8	28.4	28	26.3	29	27.8
	32.4	28.9	41.5	55.6	39.7	56.2	38.2
	33	27.4	40.2	46.9	40.8	49.2	36.7
	31.6	30.2	41	57.7	39.4	66.3	41
	100	29.7	30.3	33.7	30.8	30.7	29.7
	29.7	100	30.8	26.6	30.7	28.8	29.2
	30.3	30.8	100	36.7	53.7	39.3	64.4
	33.7	26.6	36.7	100	37.7	54.8	37.3
	30.8	30.7	53.7	37.7	100	41.1	54.9
	30.7	28.8	39.3	54.8	41.1	100	37.3
	29.7	29.2	64.4	37.3	54.9	37.3	100
	58.2	29.9	29.9	31.7	30.5	32.9	29.5
	31.5	33	33.6	31.3	32.5	33.4	33.8

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	51.7	47.2	43.2	49.6	44.9	50.3	43.8
	73.2	47.3	47.6	54.1	48.5	53	47.8
	47.2	70.4	52.7	49.4	48.2	53.9	49.2
	52	47.5	64.5	76.2	60.3	77.7	59.9
	53.4	48.7	60.3	69.4	58	68	58.4
	52	51	62.2	78.1	62.4	82.9	62
	100	50.1	49.9	53.3	49.2	52.7	50.3
	50.1	100	52.9	49.6	53.4	47	49.6
	49.9	52.9	100	58.6	76.6	61.2	81.1
	53.3	49.6	58.6	100	59.1	76.4	59.2
	49.2	53.4	76.6	59.1	100	61.7	74.4
	52.7	47	61.2	76.4	61.7	100	56.7
	50.3	49.6	81.1	59.2	74.4	56.7	100
	77.8	46.5	48.4	51.9	46.4	51.2	47.5
	49.3	54.7	54.7	50.3	50.6	52.3	52.2

fdpv1	fdpv2
31.4	28.4
51.3	31.1
29.9	34.7
31.6	31.5
32.7	31.4
32.6	33.4
58.2	31.5
29.9	33
29.9	33.6
31.7	31.3
30.5	32.5
32.9	33.4
29.5	33.8
100	31.3
31.3	100

fdpv1	fdpv2
51.9	46.9
73.2	47.5
45.6	55
49.5	48.1
52.4	47.6
52.5	52.1
77.8	49.3
46.5	54.7
48.4	54.7
51.9	50.3
46.5	50.6
51.2	52.3
47.5	52.2
100	47.5
47.5	100

Identity [E6.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	21.5	24	16.6	13.9	22
cpv01	21.5	100	29.4	27.5	26.1	24.5
cpv02	24	29.4	100	27.8	29	29.3
cpv03	16.6	27.5	27.8	100	59.6	65.1
cpv04	13.9	26.1	29	59.6	100	53.9
cpv05	22	24.5	29.3	65.1	53.9	100
cpv06	23.1	37.7	28.9	22.6	30.3	26.8
cpv07	20	28.3	46.8	24.4	25.3	23.5
cpv08	21.4	31.1	26.2	33.3	35.6	33.1
cpv09	21.4	24.6	28	73.5	58.9	64.5
cpv10	14.1	29.6	28.4	35.5	36	36.4
cpv11	18.7	24.1	28	67.3	52.8	66.2
cpv14	20	30.1	28.8	36.6	42.4	38.2
fdpv1	21	37.4	30.1	29.4	31.6	26.8
fdpv2	22.5	25.2	24.8	29.7	30.6	34.2

Similarity [E6.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	41.9	34.3	33.7	25	36.2
cpv01	41.9	100	41.2	48.5	44.1	47.9
cpv02	34.3	41.2	100	50.6	45.1	47.8
cpv03	33.7	48.5	50.6	100	79.5	83.6
cpv04	25	44.1	45.1	79.5	100	76.3
cpv05	36.2	47.9	47.8	83.6	76.3	100
cpv06	37	62.3	52.3	43.5	51.6	43.9
cpv07	34.1	47.2	66.9	38.1	46.2	42.6
cpv08	39.9	51.4	40.9	58.5	58.3	56.4
cpv09	33.2	50.3	47.8	86.8	78.1	85.5
cpv10	30.2	52.6	40.2	50.6	50	55.2
cpv11	33.2	47.1	43.5	78.6	70.4	80.6
cpv14	31.2	50	43.8	60.1	64.2	61.2
fdpv1	34.7	59.9	48.6	47.2	51.6	47.1
fdpv2	36	46	41	52.9	51.9	53.9

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14
23.1	20	21.4	21.4	14.1	18.7	20
37.7	28.3	31.1	24.6	29.6	24.1	30.1
28.9	46.8	26.2	28	28.4	28	28.8
22.6	24.4	33.3	73.5	35.5	67.3	36.6
30.3	25.3	35.6	58.9	36	52.8	42.4
26.8	23.5	33.1	64.5	36.4	66.2	38.2
100	32.2	28.4	24.6	27.1	22.3	30.1
32.2	100	25.9	24.2	27	25.4	25.7
28.4	25.9	100	32.5	59.2	31.2	68.1
24.6	24.2	32.5	100	36	66	35.1
27.1	27	59.2	36	100	33.7	60.5
22.3	25.4	31.2	66	33.7	100	37.7
30.1	25.7	68.1	35.1	60.5	37.7	100
52.2	33.3	31.5	30.8	31.2	27.3	30.7
32	25.3	31.2	31.6	35.1	32.7	34.8

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14
37	34.1	39.9	33.2	30.2	33.2	31.2
62.3	47.2	51.4	50.3	52.6	47.1	50
52.3	66.9	40.9	47.8	40.2	43.5	43.8
43.5	38.1	58.5	86.8	50.6	78.6	60.1
51.6	46.2	58.3	78.1	50	70.4	64.2
43.9	42.6	56.4	85.5	55.2	80.6	61.2
100	56.2	51.6	41.9	49	38	49.3
56.2	100	43.7	40.6	46.6	41.4	47.4
51.6	43.7	100	53	78.2	50.6	80.6
41.9	40.6	53	100	51.8	84.3	62.3
49	46.6	78.2	51.8	100	48.8	74.8
38	41.4	50.6	84.3	48.8	100	59.7
49.3	47.4	80.6	62.3	74.8	59.7	100
75.4	55.1	51	47.8	47.4	42.2	52.1
53.6	42	48.1	53.9	49.7	52.1	53.6

fdpv1	fdpv2
21	22.5
37.4	25.2
30.1	24.8
29.4	29.7
31.6	30.6
26.8	34.2
52.2	32
33.3	25.3
31.5	31.2
30.8	31.6
31.2	35.1
27.3	32.7
30.7	34.8
100	32.7
32.7	100

fdpv1	fdpv2
34.7	36
59.9	46
48.6	41
47.2	52.9
51.6	51.9
47.1	53.9
75.4	53.6
55.1	42
51	48.1
47.8	53.9
47.4	49.7
42.2	52.1
52.1	53.6
100	52.4
52.4	100

Identity [E7.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	17.2	17.6	12.5	15.4	18.9
cpv01	17.2	100	31.1	41	44.4	44
cpv02	17.6	31.1	100	28.4	25.5	27.9
cpv03	12.5	41	28.4	100	78.1	79
cpv04	15.4	44.4	25.5	78.1	100	80
cpv05	18.9	44	27.9	79	80	100
cpv06	20.1	57.6	36.5	32.1	37	39.6
cpv07	16.7	32.4	69.4	25	25.6	28.3
cpv08	20.5	43.3	29.7	54.5	58.7	55.2
cpv09	19.7	46	28.8	81	82	83
cpv10	17.5	43.7	31.9	56.9	64.4	56.6
cpv11	17	45	26.9	77.1	79	78
cpv14	18.3	44.2	30.1	55	56.6	55.1
fdpv1	18.7	53.1	32	34.3	37.4	36
fdpv2	19.1	35.6	37.3	36.2	38.4	36.3

Similarity [E7.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	28.3	33.3	23.8	28.2	33.1
cpv01	28.3	100	51.5	64.8	65.7	67
cpv02	33.3	51.5	100	50.5	45.5	51
cpv03	23.8	64.8	50.5	100	88.6	87.6
cpv04	28.2	65.7	45.5	88.6	100	88
cpv05	33.1	67	51	87.6	88	100
cpv06	38.1	78.8	58.7	61.3	63	63.4
cpv07	35.4	53.3	84.7	44.8	42.1	56.6
cpv08	35.6	60.6	49.5	68.2	70.2	73.3
cpv09	34	71	51	89.5	93	91
cpv10	35	62.1	54	71.6	77.9	75.5
cpv11	34	71	50	88.6	91	90
cpv14	33.3	65.4	54	69.4	74.5	69.2
fdpv1	38.1	73.5	56	56.2	59.6	59
fdpv2	36.2	53.8	58.8	57.1	58.6	56.9

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	20.1	16.7	20.5	19.7	17.5	17	18.3
	57.6	32.4	43.3	46	43.7	45	44.2
	36.5	69.4	29.7	28.8	31.9	26.9	30.1
	32.1	25	54.5	81	56.9	77.1	55
	37	25.6	58.7	82	64.4	79	56.6
	39.6	28.3	55.2	83	56.6	78	55.1
	100	30.6	38.5	40.6	38.5	37.6	41
	30.6	100	29.8	27	30.5	24.8	29.9
	38.5	29.8	100	60	73.1	57.1	80
	40.6	27	60	100	61.5	86	58.5
	38.5	30.5	73.1	61.5	100	60.6	69.2
	37.6	24.8	57.1	86	60.6	100	54.7
	41	29.9	80	58.5	69.2	54.7	100
	64.9	26.4	36.5	40	37.9	37	37.5
	38	37.5	41.3	38.6	43.4	37.6	41.1

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	38.1	35.4	35.6	34	35	34	33.3
	78.8	53.3	60.6	71	62.1	71	65.4
	58.7	84.7	49.5	51	54	50	54
	61.3	44.8	68.2	89.5	71.6	88.6	69.4
	63	42.1	70.2	93	77.9	91	74.5
	63.4	56.6	73.3	91	75.5	90	69.2
	100	53.7	58.7	66.3	62.5	64.4	60
	53.7	100	48.2	46.8	49.2	45.1	52.1
	58.7	48.2	100	72.4	91.3	75.2	91.4
	66.3	46.8	72.4	100	76.9	96	73.6
	62.5	49.2	91.3	76.9	100	76.9	88.5
	64.4	45.1	75.2	96	76.9	100	74.5
	60	52.1	91.4	73.6	88.5	74.5	100
	81.4	48.1	59.6	64	59.2	64	61.5
	58	55.8	58.7	62.4	57.5	57.4	58.9

fdpv1	fdpv2
18.7	19.1
53.1	35.6
32	37.3
34.3	36.2
37.4	38.4
36	36.3
64.9	38
26.4	37.5
36.5	41.3
40	38.6
37.9	43.4
37	37.6
37.5	41.1
100	35.4
35.4	100

fdpv1	fdpv2
38.1	36.2
73.5	53.8
56	58.8
56.2	57.1
59.6	58.6
59	56.9
81.4	58
48.1	55.8
59.6	58.7
64	62.4
59.2	57.5
64	57.4
61.5	58.9
100	57.6
57.6	100

Identity [L1.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	48	47.5	52	50.5	53.1
cpv01	48	100	50.6	53.8	54.7	54
cpv02	47.5	50.6	100	47.3	48.4	50.4
cpv03	52	53.8	47.3	100	71.1	81.7
cpv04	50.5	54.7	48.4	71.1	100	69.6
cpv05	53.1	54	50.4	81.7	69.6	100
cpv06	47	71.3	49.4	52.2	51.8	53.3
cpv07	45.1	50.4	72.4	46.5	47.9	48.6
cpv08	52.2	53.6	51	63.1	63.1	62.9
cpv09	50.5	55.1	49.1	78.1	69.8	79.9
cpv10	53	54.4	51.9	62.3	63.8	61.7
cpv11	51.8	51.8	48.8	76.1	66.5	80.9
cpv14	49.7	53.3	49.8	58	60.6	59.1
fdpv1	45.3	70.8	46.9	49.1	50.4	51.2
fdpv2	50	54.3	52.5	57.9	58.9	60.3

Similarity [L1.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	99.8	72.6	69.8	75.8	74.3	75.9
cpv01	72.6	100	72.9	77.8	76.6	76.2
cpv02	69.8	72.9	100	70.7	73.9	73.3
cpv03	75.8	77.8	70.7	100	86.9	92.4
cpv04	74.3	76.6	73.9	86.9	100	87.3
cpv05	75.9	76.2	73.3	92.4	87.3	100
cpv06	72.2	87.4	72.2	77.6	75.3	77.5
cpv07	70.2	74.8	89.9	71.6	73.4	73.3
cpv08	75.2	76.8	73.2	82.3	82.2	81.9
cpv09	74.9	78.1	70.8	92.6	87.9	92.2
cpv10	77.8	76.5	73.2	82.5	83	81.2
cpv11	77	77.4	73.6	92.2	85.9	92
cpv14	74.6	75.6	71.4	80.1	81.9	79.7
fdpv1	69.6	83	69.2	72.1	71.9	72.5
fdpv2	73.8	77.3	75.3	81.4	79.3	81.8

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	47	45.1	52.2	50.5	53	51.8	49.7
	71.3	50.4	53.6	55.1	54.4	51.8	53.3
	49.4	72.4	51	49.1	51.9	48.8	49.8
	52.2	46.5	63.1	78.1	62.3	76.1	58
	51.8	47.9	63.1	69.8	63.8	66.5	60.6
	53.3	48.6	62.9	79.9	61.7	80.9	59.1
	100	50.3	52.6	52.6	52.4	50.5	53
	50.3	100	49.9	49.2	50.6	47.5	49.9
	52.6	49.9	100	64.4	74.1	62.5	79.2
	52.6	49.2	64.4	100	63	76.7	61.1
	52.4	50.6	74.1	63	100	59.2	71.7
	50.5	47.5	62.5	76.7	59.2	100	59.3
	53	49.9	79.2	61.1	71.7	59.3	100
	73.8	47.3	51.9	50.1	50.9	47.8	51.4
	55.8	50.3	60.4	60.6	59.3	57.3	59.2

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	72.2	70.2	75.2	74.9	77.8	77	74.6
	87.4	74.8	76.8	78.1	76.5	77.4	75.6
	72.2	89.9	73.2	70.8	73.2	73.6	71.4
	77.6	71.6	82.3	92.6	82.5	92.2	80.1
	75.3	73.4	82.2	87.9	83	85.9	81.9
	77.5	73.3	81.9	92.2	81.2	92	79.7
	100	72.4	76.6	77.2	76.9	76.4	76.2
	72.4	100	75.6	74.1	75.5	73.1	73.8
	76.6	75.6	100	83	89.9	82.8	93.6
	77.2	74.1	83	100	82.6	90.8	81.5
	76.9	75.5	89.9	82.6	100	82.2	87.7
	76.4	73.1	82.8	90.8	82.2	100	81.8
	76.2	73.8	93.6	81.5	87.7	81.8	100
	85	69.6	73	72.2	71.7	71.1	72.3
	78.4	75.8	80.4	80.7	82.7	79.8	78.7

fdpv1	fdpv2
45.3	50
70.8	54.3
46.9	52.5
49.1	57.9
50.4	58.9
51.2	60.3
73.8	55.8
47.3	50.3
51.9	60.4
50.1	60.6
50.9	59.3
47.8	57.3
51.4	59.2
100	54.4
54.4	100

fdpv1	fdpv2
69.6	73.8
83	77.3
69.2	75.3
72.1	81.4
71.9	79.3
72.5	81.8
85	78.4
69.6	75.8
73	80.4
72.2	80.7
71.7	82.7
71.1	79.8
72.3	78.7
100	73.1
73.1	100

Identity [L2.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	27.8	27.6	28.4	28.2	27.8
cpv01	27.8	100	34.3	34.6	35.1	35.3
cpv02	27.6	34.3	100	36	35.7	34.3
cpv03	28.4	34.6	36	100	64.6	77.5
cpv04	28.2	35.1	35.7	64.6	100	65.1
cpv05	27.8	35.3	34.3	77.5	65.1	100
cpv06	26.6	60.5	32.3	33	33.9	35.9
cpv07	27.2	32	62.5	34.4	35.3	34.6
cpv08	27.7	36.3	34	48.8	48.6	49.2
cpv09	26.8	34.4	32.1	67	64.4	72
cpv10	27.8	34.9	35.4	48.1	45.8	49.1
cpv11	24.9	32.8	33.2	69.9	58	72.3
cpv14	27.7	35.9	34.8	48.2	46.8	47.8
fdpv1	27.7	58.7	35	34.4	34.7	36.2
fdpv2	27.6	35.2	30	35.6	35.1	34

Similarity [L2.tfa]:

	bpv1	cpv01	cpv02	cpv03	cpv04	cpv05
bpv1	100	51.6	51.5	52.5	48.9	50.5
cpv01	51.6	100	58.4	60.2	58.9	58
cpv02	51.5	58.4	100	58	58.9	55.4
cpv03	52.5	60.2	58	100	81.6	91.1
cpv04	48.9	58.9	58.9	81.6	100	81.1
cpv05	50.5	58	55.4	91.1	81.1	100
cpv06	51.4	82.7	57	58.1	58.7	59.6
cpv07	48.8	55.8	83.6	57.4	57.9	58.2
cpv08	50.7	60	60.8	68.5	68.3	69.8
cpv09	49.7	57.4	54.9	84.4	80.4	86.2
cpv10	47	56.5	58.1	65.5	66.6	68.1
cpv11	47.2	55.5	52.9	84.1	75.2	85.2
cpv14	51.5	58.7	61.6	66.5	67.6	68.2
fdpv1	51.1	80.4	60.7	60.2	59.3	60.9
fdpv2	46.2	57.9	53	53.6	55	53.3

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	26.6	27.2	27.7	26.8	27.8	24.9	27.7
	60.5	32	36.3	34.4	34.9	32.8	35.9
	32.3	62.5	34	32.1	35.4	33.2	34.8
	33	34.4	48.8	67	48.1	69.9	48.2
	33.9	35.3	48.6	64.4	45.8	58	46.8
	35.9	34.6	49.2	72	49.1	72.3	47.8
	100	33.5	35	33.2	34.4	30.4	34.8
	33.5	100	34.5	30.1	32.8	30.7	35.5
	35	34.5	100	48.6	67.4	48.6	82.4
	33.2	30.1	48.6	100	46.4	61.5	47.8
	34.4	32.8	67.4	46.4	100	44	67.6
	30.4	30.7	48.6	61.5	44	100	47.2
	34.8	35.5	82.4	47.8	67.6	47.2	100
	68	33.8	37.6	36.8	37.2	32.2	37.1
	36.8	33.2	36.5	34.2	36.4	32.3	34.3

cpv06	cpv07	cpv08	cpv09	cpv10	cpv11	cpv14	
	51.4	48.8	50.7	49.7	47	47.2	51.5
	82.7	55.8	60	57.4	56.5	55.5	58.7
	57	83.6	60.8	54.9	58.1	52.9	61.6
	58.1	57.4	68.5	84.4	65.5	84.1	66.5
	58.7	57.9	68.3	80.4	66.6	75.2	67.6
	59.6	58.2	69.8	86.2	68.1	85.2	68.2
	100	58.7	57	56	59	52.2	59.7
	58.7	100	60.2	59.2	56.3	55.1	60.1
	57	60.2	100	67.5	82.5	65.9	92.4
	56	59.2	67.5	100	67.5	78.3	69.2
	59	56.3	82.5	67.5	100	62.8	82.5
	52.2	55.1	65.9	78.3	62.8	99.8	64.5
	59.7	60.1	92.4	69.2	82.5	64.5	100
	86.9	59.3	59.4	60.2	60.1	55.2	59.4
	59.2	56	56.7	52.7	56.1	51	57.3

fdpv1	fdpv2
27.7	27.6
58.7	35.2
35	30
34.4	35.6
34.7	35.1
36.2	34
68	36.8
33.8	33.2
37.6	36.5
36.8	34.2
37.2	36.4
32.2	32.3
37.1	34.3
100	36
35.8	100

fdpv1	fdpv2
51.1	46.2
80.4	57.9
60.7	53
60.2	53.6
59.3	55
60.9	53.3
86.9	59.2
59.3	56
59.4	56.7
60.2	52.7
60.1	56.1
55.2	51
59.4	57.3
100	60.1
60.1	100

Supplement 2

PCR primers for cDNA detection

Target gene	Primer name	Sequence	begin	end	Amplimer length
CPV9 E1	Muf_E1_f	CCA CCC AAA GTC AGG AGT GT	1807	1826	286
	Muf_E1_r	TTA CAT GCT TCG CCT GTG AG	2073	2092	
CPV9 E2	Muf_E2_f	GGG CCA AAG ATG CAA TAG AA	2979	2998	233
	Muf_E2_r	TTG GTC AGT CAT GTC CGT GT	3192	3211	
CPV9 E4	Muf_E4_f	TGG AGG GTG ACC TAG AAA CAG	971	991	3030 (640 on cDNA)
	Muf_E4_r	TAC TGA CGC GGT GCA ATC	3983	4000	
CPV9 E6	Muf_E6_f	GCG CTC AGG AAT TTC ATT GT	298	317	200
	Muf_E6_r	TGA CCA CCT GTT GCG TGT AT	478	497	
CPV9 E7	Muf_E7_f	CAC CAC TTG ACA ACC TCT GG	730	749	212
	Muf_E7_r	GGG GCA GCA GAG TGA TGT AT	922	941	
CPV9 L1	Muf_L1_f	TAC TTT CCT GGG GAC AGT GG	6876	6895	202
	Muf_L1_r	TTG TTC CAC GTG TGG TGT CT	7058	7077	
CPV9 L2	Muf_L2_f	ATC CTG CGG TAT TTC GTG AG	5142	5161	250
	Muf_L2_r	TCC CTG AAC CTT TGA TCT GG	5372	5391	
CPV14 E1	Hal_E1_f	TCA CAG GCC AAA CAC GTA AA	1951	1970	287
	Hal_E1_r	CCA AGC AGA CTC ATG CAA AA	2218	2237	
CPV14 E2	Hal_E2_f	ACG CTC AGT GAC ACA AGC AG	2930	2959	194
	Hal_E2_r	CCC TTG TGG TCA ACC TGA CT	3124	3123	
CPV14 E4	Hal_E4_f	GGA GGG TGA ACT AGC AGC AG	831	850	3159 (750 on cDNA)
	Hal_E4_r	GTC TGC CTC GAT GTC CTC TC	3970	3989	
CPV14 E6	Hal_E6_f	CTC CAA GAA AGA GCG TGA GG	306	325	185
	Hal_E6_r	GCC ACC TCC CTC TGA CAT TA	471	490	
CPV14 E7	Hal_E7_f	GGC GCC ACA TTA AGA GAC AT	537	556	243
	Hal_E7_r	CAA ATC GAG ACA GGT CAG CA	760	779	
CPV14 L1	Hal_L1_f	GGT GTG ACA GGT CAC CCT CT	6323	6342	210
	Hal_L1_r	ACA GTC GCC ACC AGT CTT CT	6513	6532	
CPV14 L2	Hal_L2_f	TTC CCA CAA CAT TTT CGT CA	5048	5067	184
	Hal_L2_r	GAA GGC AGG ATC CGT TAC AA	5212	5231	